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Sir:

Enclosed is a certified copy of priority document 00203652.3 filed October 20, 2000, for the above-referenced application.

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
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See for original titl of th applicati n page 1 of the description.

Title: Modifications of Apoptin

The invention relates to the apoptotic pathway induced by Apoptin in tumor cells.

Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or malignant cells (Earnshaw, 1995, Duke et al., 1996). The terms transformed and tumorigenic will be used interchangeably herein. Apoptosis is characterised by shrinkage of cells, segmentation of the nucleus, condensation and cleavage of DNA into domain-sized fragments, in most cells followed by internucleosomal degradation. The apoptotic cells fragment into membrane-enclosed apoptotic bodies. Finally, neighbouring cells and/or macrophages will rapidly phagocytose these dying cells (Wyllie et al., 1980, White, 1996). Cells grown under tissue-culture conditions and cells from tissue material can be analysed for being apoptotic with DNA-staining agents, such as e.g. DAPI, which stains normal DNA strongly and regularly, whereas apoptotic DNA is stained weakly and/or irregularly (Noteborn et al., 1994, Telford et al., 1992).

The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995, White 1996, Levine, 1997). Changes in the cell survival rate play an important role in human pathogenesis of diseases, e.g. in cancer development and auto-immune diseases, where enhanced proliferation or decreased cell death (Kerr et al., 1994, Paulovich, 1997) is observed. A variety of chemotherapeutic compounds and radiation have been demonstrated to induce apoptosis in tumor cells, in many instances via wild-type p53 protein (Thompson, 1995, Bellamy et al., 1995, Steller, 1995, McDonell et al., 1995).

Many tumors, however, acquire a mutation in p53 during their development, often correlating with poor response to cancer therapy. Certain transforming genes of tumorigenic DNA viruses can inactivate p53 by directly binding to it (Teodoro, 1997). An example of such an agent is the large T antigen of the tumor DNA virus SV40. For several (leukemic) tumors, a high expression level of the proto-oncogene Bcl-2 or Bcr-abl is associated with a

strong resistance to various apoptosis-inducing chemotherapeutic agents (Hockenberry 1994, Sachs and Lotem, 1997).

For such tumors lacking functional p53 (representing more than half of the tumors) alternative anti-tumor therapies are under development based on induction of apoptosis independent of p53 (Thompson 1995, Paulovich et al., 1997). For this, one has to search for the factors involved in induction of apoptosis that do not need p53 and/or cannot be blocked by anti-apoptotic activities, such as Bcl-2 or Bcr-abl-like ones. These factors might be part of a distinct apoptosis pathway or might be (far) downstream of the apoptosis inhibiting compounds.

Apoptin (also called VP3, the terms will be used interchangeably herein) is a small protein derived from chicken anemia virus (CAV; Noteborn and De Boer, 1996, Noteborn et al., 1991, Noteborn et al., 1994; 1998a), which can induce apoptosis in human malignant and transformed cell lines, but not in untransformed human cell cultures. *In vitro*, Apoptin fails to induce apoptosis in normal lymphoid, dermal, epidermal, endothelial and smooth-muscle cells. However, when normal cells are transformed they become susceptible to apoptosis by Apoptin. Long-term expression of Apoptin in normal human fibroblasts revealed that Apoptin has no toxic or transforming activity in these cells (Danen-van Oorschot, 1997 and Noteborn, 1996).

In normal cells, Apoptin was found predominantly in the cytoplasm, whereas in transformed or malignant cells i.e. characterised by hyperplasia, metaplasia or dysplasia, it was located in the nucleus, suggesting that the localization of Apoptin is related to its activity (Danen-van Oorschot et al. 1997).

Apoptin-induced apoptosis occurs in the absence of functional p53 (Zhuang et al., 1995a), and cannot be blocked by Bcl-2, Bcr-abl (Zhuang et al., 1995), or the Bcl-2-associating protein BAG-1 (Danen-Van Oorschot, 1997a, Noteborn, 1996).

Therefore, Apoptin is a therapeutic compound for the selective destruction of tumor cells, or other hyperplasia, metaplasia or dysplasia, especially for those tumor cells that have become resistant to (chemo)-therapeutic induction of apoptosis, due to the lack of functional p53 and (over)-expression of Bcl-2 and other apoptosis-inhibiting lesions (Noteborn and Pietersen, 1999). It appears that even pre-malignant, minimally transformed cells are sensitive to the death-inducing effect of Apoptin. In addition, Noteborn and Zhang (1998) have shown that Apoptin-induced apoptosis could be suitable for the diagnosis of cancer-prone cells and treatment of cancer-prone cells.

The fact that Apoptin does not induce apoptosis in normal human cells, at least not *in vitro*, implies that there would be little or no toxic effect of Apoptin treatment *in vivo*. Noteborn and Pietersen (1998) and Pietersen et al. (1999) have provided evidence that adenovirus expressed Apoptin does not have an acute toxic effect *in vivo*. In addition, in nude mice it was shown that Apoptin has a strong anti-tumor activity.

However, to further enlarge the array of therapeutic anti-cancer or anti-auto-immune-disease compounds available in the art, additional therapeutic compounds are desired, especially in those cases wherein p53 is (partly) non-functional.

In a preferred embodiment, the invention provides an isolated or recombinant phosphorylated Apoptin or functional equivalent and/or functional fragment thereof. More preferably said Apoptin is tumor-specifically phoshorylated. Even more preferable said Apoptin is phosphorylated on a threonine residue, which residue in the Apoptin depicted in Figure 1 is located between amino acid 100 to 121. Most preferably Apoptin is phosphorylated on a threonine residue, which residue in the Apoptin of Figure 1 is located at amino acid 106 and/or 107. Functional equivalent and/or functional fragment thereof is herein defined as Apoptin and/or part thereof, optionally coupled to

other components, wherein phosphorylation is according to the invention or wherein phosphorylation is mimicked by, for example introducing a negative charge which mimics the negative charge of phosphate or wherein the effect of phosphorylation is obtained by other methods known in the art, for example chemical crosslinking of phosphate or phosphate mimics to Apoptin. An example of a functional equivalent, is a single point mutation of, for example, amino acids 107 from threonine to glutamic acid as depicted in Figure 4 and described in the detailed experimental part herein. An example of a functional fragment, which in this case is coupled to another component, is GFP-deletion mutant GFP-70-121 as depicted in Figure 2 and described in the detailed description herein. The fact that Apoptin phosphorylated according to the invention and evenmore is tumor-specifically phosphorylated discloses part of the apoptotic pathway as induced by Apoptin in tumor cells and opens the way to, for example, further elucidation of the apoptotic pathway induced by Apoptin in tumor cells, identification of crucial mediators of phosphorylation in human tumor cells, new diagnostic assays or new pharmaceutical compounds.

In another embodiment the invention provides a vector encoding Apoptin or functional equivalent and/or functional fragment thereof, which can be phosphorylated according to the invention and furthermore, the vector also comprises a nucleic acid molecule encoding a kinase which can provide the phosphorylation of Apoptin according to the invention.

In yet another embodiment the invention provides a gene delivery vehicle comprising a vector according to the invention which enables using Apoptin or functional equivalent and/or functional fragment thereof, phosphorylated according to the invention, for cancer treatment via the use of gene-therapy. By equipping a gene delivery vehicle with a nucleic acid molecule encoding Apoptin or functional equivalent and/or functional fragment thereof and by further providing a kinase, and by targeting said vehicle to a cell or cells that show over-proliferating behaviour and/or have shown decreased death rates, said gene delivery vehicle provides said cell or cells

with the necessary means of apoptosis, providing therapeutic possibilities.

Furthermore, the invention provides a host cell comprising a vector or a gene delivery vehicle encoding Apoptin or functional equivalent and/or functional fragment thereof; which can be phosphorylated according to the invention. Not all host cells comprising this vector are capable of protein phosphorylation and by providing a kinase Apoptin can be phosphorylated according to the invention.

The invention also provides an isolated or synthetic antibody or functional equivalent and/or functional fragment thereof specifically recognising phosphorylated Apoptin according to the invention. Such an antibody is for example obtainable by immunising an immuno-competent animal with phosphorylated Apoptin or an immunogenic fragment or equivalent thereof and harvesting polyclonal antibodies from said immunised animal, or obtainable by other methods known in the art such as by producing monoclonal antibodies, or (single chain) antibodies or binding proteins expressed from recombinant nucleic acid derived from a nucleic acid library, for example obtainable via phage display techniques. Phosphospecific antibodies are routinely generated, and in most cases an antibody can be developed that will recognize the phosphorylated epitope of the protein of interest, but not the non-phosphorylated version (Blaydes et al., 2000).

With such an antibody, the invention also provides an immunoassay comprising an antibody according to the invention. A lot of immunoassays are available within the art, for example ELISA (Enzyme Linked Immuno Sorbent Assay) or Western blotting.

Furthermore, the invention provides use of Apoptin or functional fragment thereof which can be phosphorylated according to the invention for diagnostic purposes. One embodiment of a diagnostic assay is a method for detecting the presence of cancer cells or cells that are cancer prone in a sample of cells comprising providing a cell lysate of said cells to Apoptin or functional fragment thereof which can be phosphorylated according to the invention and

determining phosphorylation state of said Apoptin. Another embodiment of a diagnostic assay is a method for identifying a putative cancer-inducing agent comprising submitting a sample of cells to said agent, and detecting the presence of cancer cells or cells that are cancer prone in a sample of cells comprising providing a cell lysate of said cells to Apoptin or functional fragment thereof which can be phosphorylated according to the invention and determining phosphorylation state of said Apoptin. Another embodiment of a diagnostic assay is a method for testing the *in vitro* treatment effect of Apoptin on tumor cells comprising submitting a cell lysate of said tumor cells to said Apoptin or functional fragment thereof which can be phosphorylated according to the invention and determining phosphorylation state of said Apoptin. In all these examples Apoptin provided to the cells would preferably be non-phosphorylated Apoptin. After incubation with, for example cell lysates the phosphorylation state can be detected according to a method described herein. Even more preferably Apoptin comprises a protein fusion. Protein fusion are well known in the art and can be N- terminal and/or C-terminal linked, examples are histidine fusions or maltose binding protein fusions. An example of an Apoptin protein fusion is His-VP3 as described in the detailed description herein.

In another embodiment the invention comprises a kit for detecting the presence of cancer cells or cells that are cancer prone or a kit for testing the *in vitro* treatment effect of Apoptin on tumor cells. Such a kit comprises an antibody according to the invention.

An example of a kit is described in more detailed below:

The fact that lysates of tumor or transformed cells are able to phosphorylate Apoptin *in vitro* forms the basis for a rapid, non-subjective, low-labour-intensive diagnostic kit that should be able to determine whether a patient's biopsy is cancerous, or, more importantly for early therapeutic intervention, cancer-prone. An advantage of such a method is that one does not need to culture primary (tumor) cells under tissue-culture conditions, which in

many cases proves to be difficult or impossible. A kit would be divided into, for example, three components:

1) Patient biopsy sample (provided by a doctor)

2) Biopsy lysis and phosphorylation tube, to which the sample is added and incubated

3) ELISA-based system for rapid detection of Apoptin phosphorylation

As an example, a more detailed description of the invention:

1) The sample is straightforward; only a very small amount of suspect material is required. In addition, as a control, the doctor should also take some normal cell material from the same patient; for example, a brief, non-invasive, non-painful scraping of the inside of a patient's cheek would provide a rich source of epithelial cells for analysis. Finally, the kit will also provide a standardized lysate of transformed cells as a positive control.

2) The sample is added to the provided lysis tube that already contains a combination kinase incubation/lysis solution optimised for phosphorylation of Apoptin. This will comprise, in general, of a mild detergent, a buffer, physiological salts, protease inhibitors, phosphatase inhibitors, inorganic phosphate, and recombinant Apoptin protein. The sample is simply added to the tube, mixed briefly and then incubated for 15-30 minutes at 30°C. If the sample of the patient is transformed or tumorigenic, the resident kinase should phosphorylate the recombinant Apoptin in the tube, as should the positive control standard lysate, but not negative control lysate derived from the patient's cheek epithelia.

3) ELISA (enzyme-linked immunosorbant assay) is a technique widely used in diagnostic kits (e.g., pregnancy tests, HIV tests) that exploits the ability of a specific antibody to bind to the desired antigen; for detection, the antibody is used in combination with a colorimetric readout such that a color appearance or change indicates a positive result, whereas no appearance or change indicates a negative result. Note that the ELISA assay can be performed in a variety of ways, and below is only one example of how it might be performed.

Descriptions and protocols for various ELISA strategies are presented in Harlow and Lane, 1988.

An antibody, according to the invention, specific for a phosphorylated epitope of Apoptin, can be used in this kit. The ELISA will occur on a solid substrate (e.g. multiwell plate). Attached to the solid substrate will be the phospho-Apoptin-specific antibody. The wells will be hydrated with wash solution, treated with blocking solution, then the contents of the Lysis tubes (sample(s), and controls) will be added to the wells. Presumably multiple patient samples could be batch-assayed in parallel; many diagnostic ELISAs feature convenient modular well strips that be broken off to the number required. In addition, the kit will provide positive and negative controls for the ELISA itself (for example, phospho-Apoptin peptide solution, nonphosphorylated Apoptin peptide solution and saline solution). After a brief incubation, allowing any phosphorylated Apoptin generated by the kinase in the patients' biopsy to bind to the specific antibody, the rest of the lysate is washed away thoroughly. In particular, all of the non-phosphorylated Apoptin will be removed, which is key for the next step. Now, all the wells will receive a droplet of, for example, the anti-Apoptin monoclonal 111.3, which recognizes Apoptin regardless of phosphoryation state. This version of 111.3 will be pre-conjugated with, for example, the enzyme alkaline phosphatase, which is able to convert the substrate nitrophenyl phosphate into a bright yellow color detectable by eye as well as by a multiwell spectrophotometer. Other combinations of enzymes and substrates are also available (see Harlow and Lane, 1988 for details). After incubation, allowing 111.3 to bind to the phosphorylated Apoptin captured by the phospho-specific antibody attached to the solid substrate, the wells are washed again. Finally, the enzyme substrate solution is added, and the color is allowed to develop and the reaction is stopped. A color change indicates the presence of phosphorylated Apoptin, which in turn infers the presence of a tumor/transformed-specific kinase activity in the patient's sample.

Confirmatory Support protocol:

ELISA can sometimes produce false-positive results. In the case of serious illnesses such as HIV, a positive ELISA result is then grounds for a more precise, labour intensive assay to confirm the ELISA result. In the case of the standard HIV ELISA, for example, a positive result is then confirmed by Western Blot analysis. Such a confirmatory protocol would also be appropriate for a cancer diagnostic. A Western blot using the phospho-specific Apoptin antibody is also novel and forms also part of the invention. In conjunction, the doctor could also perform more standard pathology tests.

The diagnostic kit as described above, in addition to clinical applications, can be useful as part of a method to identify a mediator of tumor-specific phosphorylation (for example a kinase). For example, one way to arrive at such a mediator is to enable or inhibit the phosphorylation and uses the kit as a screening tool.

Furthermore the invention provides a pharmaceutical composition comprising phosphorylated Apoptin, a vector or a gene delivery vehicle according to the invention.

Such a pharmaceutical composition is in particular provided for the induction of apoptosis, for example, wherein said apoptosis is p53-independent, for the treatment of a disease where enhanced cell proliferation or decreased cell death is observed, as is in general the case when said disease comprises cancer or auto-immune disease. Herewith the invention provides a method for treating an individual carrying a disease where enhanced cell proliferation or decreased cell death is observed comprising treating said individual with a pharmaceutical composition according to the invention.

The invention will be explained in more detail in the following description, which is not limiting the invention.

EXPERIMENTAL PART

Plasmids

Description of pCMV-Apoptin and pCMVneo

The plasmid pCMV-Apoptin, which encodes a naturally occurring form of Apoptin, was described previously by Danen-Van Oorschot et al. (1997). In short, the plasmid pCMV-Apoptin contain the human cytomegalovirus (CMV) promoter and CAV DNA sequences (nt 427-868) encoding Apoptin exclusively. The synthesized Apoptin protein harbours apoptotic activity and is identical to GenBank Q99152 except position 116 contains a K > R change. The empty vector pCMV-neo was described by Baker et al. (1990) and is used as a negative control. Figure 1 shows the amino acid sequence of the Apoptin protein with its domains and potential phosphorylation sites indicated.

Construction of N-terminal deletion mutants of Apoptin fused to GFP

N-terminal deletion mutants of Apoptin were fused C-terminally to GFP in the vector phGFPS65T obtained from ClonTech (USA). Briefly, GFP containing an activating mutation is juxtaposed, via a 4 amino acid tether, to full-length Apoptin (GFP-VP3) or portions thereof: GFP-1-69, GFP-70-121, GFP-80-121 and GFP-100-121, where the numbers indicate the amino acid residues of Apoptin included in the construct. The GFP-VP3 fusion genes are under the regulation of the SV40 promoter, which is active in a broad range of mammalian cell types. Figure 2 depicts the GFP mutants in schematic form.

For the construction of pGFP-VP3, the *Nde*I-*Bam*H1 fragment of the plasmid pGBT9-VP3 (Noteborn and Danen-Van Oorschot, 1998) was isolated and cloned in the *Bsr*GI and *Not*I sites of the linearized phGFPS65T plasmid.

For the construction of pGFP-1-69 and pGFP-70-121, respectively, the *Nde*I-*Bam*H1 fragment of plasmid pGB9-VP3 was treated with restriction enzyme *Bsr*I. The *Bsr*GI-*Bsr*I fragment and the required *Bsr*GI-*Nde*I and *Bsr*I-

*Not*I linkers were cloned in the *Bsr*GI-*Not*I-treated pGFPS65T plasmid resulting in pGFP-1-69 plasmid vector, whereas the *Bsr*GI-*Bam*H1 fragment and the required *Bam*H1-*Not*I linker were cloned in the *Bsr*GI-*Not*I-treated pGFPS65T plasmid resulting in GFP-70-121.

For the construction GFP-80-121, a PCR DNA fragment encoding the amino acids 80-121 of the Apoptin gene was produced. The phGFPS65T plasmid and the PCR fragment were digested with the restriction enzymes *Bsr*GI and *Not*I. Subsequently, the cleaved PCR fragment was cloned in the CIP-treated phGFPS65T plasmid.

For the construction of GFP-100-121, a PCR DNA fragment encoding the amino acids 100-121 of the Apoptin gene was produced. The phGFPS65T plasmid and the PCR fragment were digested with the restriction enzymes *Bsr*GI and *Not*I. Subsequently, the cleaved PCR fragment was cloned in the CIP-treated phGFPS65T plasmid.

Construction of alanine mutants

A series of 5-Alanine (Ala(5)) scanning mutants of the Apoptin gene was a kind gift from Dr D. Mumberg from Schering AG, Berlin. First, an Apoptin DNA was constructed containing additional unique restriction enzyme sites that allow for ease of cloning of systematic Ala(5)-mutants. Then, sequential stretches of 5 amino acids of Apoptin were systematically exchanged by 5 Ala residues each using a linker substitution strategy. The Ala(5)-mutants have been sequenced and cloned in a modified expression plasmid vector pIRESneo (ClonTech, USA) under the control of the CMV promoter. The relevant Ala(5)-mutants of Apoptin are shown in Figure 3.

Construction of threonine and proline replacement constructs

Further, we received (a kind gift from Schering, Berlin) a number of threonine replacement mutants of Apoptin, and one proline replacement mutant, either using alanine to eliminate potential phosphorylation or

glutamic acid to mimic constitutive phosphorylation. The strategy used was the same as for the Ala(5)-mutants (see above) except that the linkers contained the appropriate point mutations. These constructs are depicted in Figure 4.

Cloning of the His-tagged vp3 construct

Vp3 lacking a stop codon was cloned in the *Nde*I site and *Not*I site of the IPTG-inducible bacterial expression plasmid pET22b, which provides in frame a 6-histidine tag and a stop codon. The essential regions of the final pVp3H6 DNA construct were confirmed.

All cloning steps were performed essentially according to Maniatis et al. (1982) and sequencing of all constructs was based on the method described by Sanger et al. (1977) at Baseclear, Leiden or at Schering, Berlin.

Vp3H6 expression and purification

The Vp3H6 construct was transformed in BL21(DE3) bacteria (Novagen) and a colony was grown at 37°C to an OD600 of ca. 0.6. Expression was then induced by adding 1 mM IPTG and the cells were grown for an additional 3 hrs. After harvesting by centrifugation, the cells were lysed in a Bead-Beater (Biospec Inc.) in lysis buffer (containing 50 mM NaHEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and protein inhibitors (Complete, Boehringer)). The inclusion bodies were harvested by centrifugation and made soluble by suspending in Solubilisation Buffer (containing 50 mM HEPES pH 7.4, 20 mM Glycine, 1 mM EDTA, 10 mM DTT, 8 M Urea). The cleared supernatant was loaded directly on UNO-S12 (Biorad) pre-equilibrated with: 20 mM KPO_4, 5 mM Imidazole, 6 M urea, 1 mM GSH. The His-tagged VP3 protein (Vp3H6 protein) was eluted with an NaCl gradient (0-1 M NaCl at 3 ml/min with a total volume of 200 ml). Vp3H6 was eluted between 400 and 650 mM NaCl. It was loaded directly on Ni-NTA (Qiagen) (pre-equilibrated in 20

mM KPO_4 pH 7.4, 5 mM Imidazole, 500 mM NaCl, 6 M urea at 4C). Next, the column was washed with 20 mM KPO_4 pH 7.4, 20 mM Imidazole, 500 mM NaCl, 6 M GuHCl. The GuHCl was removed by washing with 20 mM KPO_4 pH 7.0, 400 mM NaCl, 2 mM MgCl2, 1 mM GSH, and Vp3H6 protein was eluted with 20 mM KPO_4 pH 7.4, 400 mM NaCl, 500 mM Imidazole, 2 mM MgCl2. The Vp3H6 protein containing peak fractions were pooled and 5 mM EDTA was added to remove nickel traces. The sample was dialysed (1 volume to 200) to 20 mM KPO_4 pH 6.5, 400 mM NaCl, 2 mM MgCl2, 1 mM DTT. Finally, the Vp3H6 protein was concentrated on Centricon YM3 filters (Millipore) to at least 7 mg/ml.

Cell lines and culturing

The following established cell lines have been described previously: Saos-2 human osteosarcoma cells (Diller et al. 1990), which are functionally deficient for p53 function; U2OS human osteosarcoma cells (Diller et al. 1990), which are functionally competent for p53 function; VHSV (Danen-Van Oorschot et al., 1997) are SV40-large-T transformed VH10 human fibroblasts, SVK14 (Danen-Van Oorschot et al., 1997) are SV40-large-T-transformed keratinocytes, H1299 (Friedlander et al., 1996) are human lung cell carcinoma cells; primary passage 2 human keratinocytes were a gift from Dr. M. Ponec, Department of Dermatology, Leiden University Medical Center; low-passage primary human fibroblasts (VH10) were a gift from Dr. L. Mullenders, Leiden University Medical Center, Department of Chemical Mutagenesis and Radiation Genetics.

All cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, and cultured at 10% CO_2 in a humidified 37°C incubator, except for the primary keratinocytes, which were grown in DMEM/Hams F12 (3:1), supplemented with 5% bovine calf serum (Hyclone), 10^{-6} M isoproleronol, 10^{-6} M

hydrocortisone, 10^{-7} M insulin, and penicillin/streptomycin and cultured at 37° C at 7.5% CO_2.

Transfections

For biochemical analyses, cells were plated the day before on 10 cm dishes such that cultures were 40% confluent at the time of transfection. Seven μg DNA was transfected using a 3:1 (μl:μg DNA) ratio of FuGene 6 (Roche) according to the manufacturer's instructions. The complexes were incubated on the cells in the presence of full serum and were left on until the cells were assayed. For apoptosis immunofluorescence assays, $0.5x10^5$-$1x10^5$ cells were plated on 2-well Permanox chamber slides (Nunc) and transfected the same as for 10 cm plates, except that only 1.5 μg DNA per well was used and the rest of the transfection components were scaled down accordingly.

In vivo orthophosphate metabolic labelling assay

Forty-eight hours post-transfection, cultures transfected with plasmids encoding Apoptin, mutant Apoptin, or controls were washed two times with phosphate-free DMEM (PFD; Sigma), then incubated for ten minutes in PFD in the tissue culture incubator to deplete intracellular phosphate. Next, the plates were incubated with PFD supplemented with ^{32}P-orthophosphate (0.5-1.25 mCi/ml) for four hours. Cells were washed with ice-cold PBS, then lysed in 1 ml RIPA buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 % SDS, 1 % NP-40, 1 % sodium deoxycholate, supplemented with the following protease or phosphatase inhibitors at standard concentrations: trypsin inhibitor, pepstatin, leupeptin, aprotinin, PMSF, β-glycerophosphate, sodium vanadate, and sodium fluoride). Lysates were incubated on ice for 30 minutes, centrifuged for 10 minutes at 13,000 rpm in a refrigerated microfuge, and the supernatants were immunoprecipitated with affinity-purified polyclonal rabbit serum (VP3-C) raised against the C terminal portion of Apoptin and protein A beads using standard methodology. The final pellet was resuspended in 2x

denaturing Laemmli buffer and stored at –20°C until processing.

Immunoprecipitation samples were divided into two aliquots, one consisting of 2 µl and the other of the remainder (38 µl). Each set was run on parallel 15% SDS-PAGE gels; the 38 µl gel sets were dried, whereas the 2 µl gel sets were Western-transferred to Immobilon membranes (see below). The dried gels containing the bulk of the immunoprecipitates were subjected to autoradiography to visualize phosphorylated protein, whereas the parallel membranes were immunoprobed to confirm the presence of Apoptin in the lysates, regardless of phosphorylation state. In some cases, the 38 µl gels were not fixed but the entire experiment was transferred to Immobilin via Western blotting, autoradiography was performed on the Immobilin and then the membrane was Western-immunoprobed for precise overlaying of the radioactive signal with the Western signal. All autoradiographs were exposed along with fluorescent marks to facilitate subsequent orientation for band isolation (see below).

Western blot analysis

Protein was electroblotted from gel to PVDF membranes (Immobilin, Millipore) using standard techniques. Membranes were blocked in a tris-buffered saline solution supplemented in 0.5% Tween-20 (TBS-T) and 5% non-fat dry milk (TBS-TM) for 30 minutes, washed briefly in TBS-T, then incubated for 1 hr at room temperature with primary antibody, depending on the experiment, in TBS-TM at the following concentrations: 111.3 hybridoma supernatant (monoclonal recognizing the N-terminus of Apoptin) at 1:25; VP3-C purified serum at 1:3; or anti-GFP (Living Colors Peptide Antibody, Clontech) under conditions recommended by the manufacturer. After 3x 5' washes in TBS-T, membranes were further incubated in the appropriate antibody (anti-mouse Ig, anti-rabbit Ig,) or Protein A (for GFP mutants of a size that co-migrates with contaminating heavy-chain immunoglobulin) conjugated to horseradish peroxidase (HRP). After 3x 20' washes in TBS-T,

membranes were subjected to enhanced chemiluminescence using standard techniques, exposed to x-ray film (Kodak), and films were developed using standard automated methods.

Phosphoamino acid analysis (PAA)

Using exposed autoradiography films for orientation, bands visualised in the ortholabelling experiments (above) corresponding to phosphorylated Apoptin (P-Apoptin) or the corresponding region in negative control lanes were excised from the PVDF membranes using a razor blade. Membrane fragments were re-hydrated with methanol, blocked in 0.5% polyvinylpyrrolidone-360,000 in 100 mM acetic acid for 30' at 37°C, washed 5x with water and 2x with 0.05M NH_4HCO_3 trypsin buffer, then digested overnight with 10 µg trypsin (Tpck-treated, Worthington, USA) in trypsin buffer at 37°C to remove all proteins from the membrane. Samples were digested a second time for two hours, the supernatant was supplemented with water and split into two samples, one for PAA and one for tryptic phosphopeptide analysis (see below). PAA samples were further processed using the method of Hunter (basically as described in the text of "Protein phosphorylation. Part B: Analysis of protein phosphorylation, protein kinase inhibitors, and protein phosphatases", Methods Enzymol. 1991, 201). Briefly, the peptides were lyophilised, hydrolysed for 1 hr in 6N HCl at 110°C, lyophilised, then resuspended in pH1.9 buffer, supplemented with non-radioactive PAA standards (1.0 mg/ml of each P-Thr, P-Ser, and P-Tyr, Sigma) and spotted onto pre-coated cellulose TLC plates (Merck). On a Hunter thin-layer electrophoresis apparatus, plates were run in the first dimension at 1.5 kV for 20' in pH 1.9 buffer, dried, turned 90 degrees counter-clockwise and run again in the second dimension at 1.3 kV for 16' in pH 3.5 buffer. After drying the plate, PAA standards were visualised by spraying with 0.25% ninhydrin in acetone and baking the plates at 65°C for 10' to develop the colour. Finally, plates were exposed to PhosphoImage screens to detect the radioactive phosphoamino acids.

Tryptic phosphopeptide mapping (TPM)

Samples split off from the PAA procedure (see above) were subjected in parallel to TPM analysis using the method of Hunter (for reference see above). Briefly, samples were lyophilized, oxidized for 1 hr on ice in freshly-prepared performic acid, supplemented with water and lyophilized again. Samples were resuspended in pH1.9 buffer and loaded onto TLC plates. On a Hunter thin-layer electrophoresis apparatus, plates were run in the first dimension at 1 kV for 25' in pH 1.9 buffer, dried, then placed in the same orientation in a liquid chromatography tank with PhosphoChromo buffer overnight for the second dimension (no more than 15 hours). Plates were then dried and exposed to PhosphoImage screens to detect the radioactive phosphopeptides. Predicted trypsin cleavages were determined by the ExPASY computer program Peptide Mass.

***In vitro* phosphorylation of recombinant Apoptin**

Soluble, purified, recombinant Apoptin protein fused N-terminally to a histidine tag and produced in *E. coli* bacteria was a gift from Rutger Leliveld, Department of Chemistry, Leiden University (for cloning, production and purification see above). Proteins can only be produced in *E. coli* in an unphosphorylated state, so the His-Apoptin serves as a good substrate to test whether tumor lysates are able to phosphorylate Apoptin.

Subconfluent Saos-2 and low-passage VH10 cells were washed twice with ice-cold PBS, scraped on 0.5 ml ice cold PBS with a rubber policeman, transferred to Eppendorf tubes and centrifuged two minutes at 4000 rpm in a cold microfuge. Supernatants were removed, and 50-100 µl of kinase buffer (20 mM Hepes pH 7.2, 10 mM MgCl2, 10 mM KCl, 0.5 mM trypsin inhibitor and 0.1 mg/ml Na_3VO_4) was added. Samples were frozen-thawed three times, alternating between ethanol/dry ice and regular ice to lyse the cells, then were centrifuged at 14,000 rpm in the microfuge. The supernatants were

transferred to fresh tubes as the final cellular lysates. Protein concentration was determined at OD595 using the Bradford reagent according to standard procedures. Equal amounts of cellular lysates were incubated for 30' at 30°C with 0.5 mg of the recombinant Apoptin protein, 50 μM ATP, 0.5 μCi of γ-^{32}P-ATP, and kinase buffer to bring the total volume to 30 μl. Samples were run on SDS-PAGE gels (same procedure as described in "Western blot analysis"), which were fixed and then visualised by autoradiography. Parallel non-radioactive reactions were carried out and treated identically, except that the resultant gels were subjected to Western blot analysis with the antibody 111.3 (same as within *in vivo* section) to confirm the presence of the Apoptin protein.

Apoptosis assays

Constructs encoding Apoptin or mutants thereof, or lacZ-myc plasmid (pcDNA3.1/Myc-His LacZ, Invitrogen) as a negative control, were transfected into cells in chamber slides, and apoptosis was scored 3-5 days later as previously described (Danen-Van Oorschot et al., 1997). Briefly, cells were fixed with 80% acetone, immunostained using 111.3 as a primary antibody for Apoptin (mutants), or 9E10 anti-myc monoclonal (Evan et al,1985) for the lacZ-myc control, using FITC-conjugated goat-anti-mouse Ig as a secondary antibody, and counterstained with DAPI. Slides were coded by an individual not performing the scoring, mounted in DABCO/glycerol with coverslips and inspected by fluorescence microscopy. Only positive cells were assessed for apoptosis using nuclear morphology as the criterion. At least 100 cells per well were scored and the assays were done multiple times.

Results

1. *In vivo* tumor-specific phosphorylation of Apoptin

In order to determine whether a phosphorylation event is responsible for the tumor-specificity of Apoptin-induced apoptosis, we first investigated Apoptin expressed in tumor (Saos-2) and normal (VH10) cells, which have been extensively characterized with regards to Apoptin localization and killing in previous reports (reviewed by Noteborn, 1999). The cultures were transfected with pCMV-Apoptin or the empty vector plasmid (pCMVneo), labelled *in vivo* with ^{32}P-orthophosphate, immunoprecipitated with anti-Apoptin VP3-C polyclonal antisera, and then the proteins were resolved by SDS-PAGE. After autoradiography, an approximately 16 kD phosphoprotein of a size consistent with Apoptin was detected in Saos-2 cells transfected with pCMV-Apoptin, but was absent in Saos-2 cells transfected with empty vector. However, the phosphoprotein was not detected in non-tumorigenic VH10 cells transfected with pCMV-VP3. In a parallel Western blot analysis using the 111.3 anti-Apoptin monoclonal antibody, we confirmed that Apoptin is strongly detected in all cells in which pCMV-Apoptin was transfected, including the VH10 cells.

These results indicate that Apotin is phosphorylated in tumor Saos-2 cells but not in normal VH10 cells.

In order to confirm that this tumor-specific phosphorylation was not merely due to the cell-type difference between osteosarcoma cells and fibroblasts, the ortholabelling assay was repeated with several other cell types. It has been shown previously that VH10 cells, when transiently or stably transformed with SV40 large T antigen (LT), become concomitantly sensitive to Apoptin-induced apoptosis (Noteborn et al., 1998b). The same is true for primary keratinocytes. Thus, we first investigated whether this transformation state correlated with phosphorylation state of Apoptin, by comparing VH10- and passage 2 keratinocyte-expressed Apoptin to Apoptin expressed in corresponding SV40 LT transformed cell lines VHSV and SVK14, respectively. Only the transformed cells exhibited phosphorylated Apoptin,

despite the fact that Apoptin was robustly immunoprecipitated from all cells that were transfected with pCMV-VP3, as assessed by Western blot analysis. Similarly, Apoptin was also shown to be phosphorylated in U2OS osteosarcoma cells and in H1299 human lung carcinoma.

In conclusion, we have shown that Apoptin phosphorylation, in common with its ability to translocate to the nucleus and induce p53-independent apoptosis, is dependent on the transformed or tumorigenic state of the cell.

2. Tumor lysates but not normal lysates can phosphorylate recombinant Apoptin *in vitro*.

To confirm our *in vivo* results on tumor-specific phosphorylation of Apoptin, we performed an *in vitro* kinase assay in the presence of γ-^{32}P-ATP using mild cellular lysates from tumor cells or normal cells as a kinase donor, and recombinant, bacterially produced Apoptin as a substrate. This experiment showed that whereas Saos-2 lysates were able to phosphorylate recombinant Apoptin *in vitro*, lysates from normal VH10 cells could not.

These data are consistent with the *in vivo* results, and also suggest that the tumor-specific phosphorylation seen *in vivo* was the result of a tumor-specific kinase.

3. Apoptin is phosphorylated in a tumor-specific manner on threonine(s).

In order to determine whether tumor-specific phosphorylation of Apoptin is necessary and sufficient for apoptotic activity, we first had to map the relevant site(s). Although Apoptin is a small protein, it contains a large number of potential phosphorylation sites (12 serines, 14 threonines, and one tyrosine, or roughly one-quarter of the protein). A schematic drawing showing the potential phophorylation sites is depicted in Figure 1. To narrow down the possibilities, Saos-2 cells were transfected with pCMV-Apoptin or pCMVneo (negative control) and a phosphoamino acid analysis was performed on

phosphorylated Apoptin (P-Apoptin) derived from Saos-2 cells to determine whether the modification occurs on threonine, serine or tyrosine residues. We determined that phosphorylation occurs specifically on threonine residues. There was also a fainter phosphoserine signal evident, but as this was also evident in samples corresponding to Saos-2 cells transfected with empty vector and, indeed, in irrelevant cells (e.g., mock-transfected VH10 cells), we conclude that this phosphoserine signal was probably derived from a co-migrating cellular phosphoprotein contaminant, and thus was not significant. However, we could not rule out absolutely the presence of a minor, specific phosphoserine signal that is obscured by the background. This result was reproduced in a second tumor cell line (U2OS). In all performed experiments, no indication was found for a phosphorylation event on the tyrosine amino acid.

These data suggest that Apoptin is tumor-specifically phosphorylated on one or more of the 14 threonine residues. However, the presence of a minor, specific phospho-serine signal cannot be ruled out.

4. Phosphorylation of Apoptin occurs between residues 100-121.

In order to narrow down further the region on Apoptin where the phosphorylation occurs, we assayed a series of gross N-terminal deletion mutants of GFP-Apoptin (depicted in Figure 2). U2OS cells were transfected with the N-terminal deletion mutants of GFP-Apoptin and an *in vivo* labeling assay (as described before) was performed. GFP alone exhibited a very faint background phosphorylation in this assay, but in contrast, full-length Apoptin fused to GFP was strongly phosphorylated. GFP-1-69, which encodes the N-terminal half of Apoptin, showed only background phosphorylation. In contrast, all the C-terminal fragments were specifically phosphorylated (GFP-70-121, -80-121, and 100-121) *in vivo*. GFP-100-121 was somewhat less phosphorylated than the others, but still significantly so. Nevertheless, there

are no threonine differences between residues 80-100, so the result using the 80-121 mutant should be functionally equivalent in the assay.

In conclusion, a tumor-specific phosphorylation site or sites are likely to reside downstream of residue 100, in a region that contains only four threonines: a triple-T stretch at positions 106-108, and a single T at position 114.

5. Only one major tryptic fragment of Apoptin is phosphorylated *in vivo*

An inspection of the computer predicted trypsin digest pattern (ExPASY program Peptide Mass) of Apoptin revealed that the T cluster at 106-108 and the single T at position 114 reside on different tryptic peptides. In order to distinguish between these two loci, we performed tryptic phosphopeptide mapping of P-Apoptin derived from Saos-2 cells. We found that only one major tryptic fragment was phosphorylated. On a very long exposure, several other faint spots were also detected, but because the signal ratio of the major spot is so extremely high compared to the faint spots, they probably do not represent relevant sites, or alternatively may be derived from the proposed cellular contaminant also seen in PAA.

With this assumption, taken together with the GFP-deletion mutant results, the presence of only one major phosphorylated peptide suggests that Apoptin is phosphorylated either within the triple-T stretch or on the lone T, but not in both loci.

6. Phosphorylation occurs within the Triple-T locus (106-108).

In order to pinpoint finally which loci contained the phosphorylation site, as well as to rule out the formal possibility of minor involvement of other residues between position 80 and 121, we used the *in vivo* phosphorylation assay to analyze a series of 5-alanine scanning mutants, which encode a protein that has every 5 residues in the Apoptin gene replaced by five alanines

(Figure 3). Of particular interest were mutants Ala(5)-106, in which T106-108 are all replaced by alanines, and Ala(5)-111, in which T-114 is also replaced. We tested all mutants between 86-115 in the *in vivo* phosphorylation assay. (Unfortunately, the mutants between 80 and 85 could not be tested in the assay because this mutation abolishes the immunoprecipitation epitope). Some of the mutants migrated as doublets. Because the lowest band was closer in migration to wild-type Apoptin and was also universally present, we assumed that the lower band was the significant band. In these experiments, all constructs were expressed at roughly equal levels, as determined by immunostaining the PVDF membrane post-autorad analysis. Most of the tested alanine mutants tested were robustly phosphorylated, including Ala(5)-111, suggesting that the lone T at position 114 is not involved in phosphorylation. In contrast, the Ala(5)-106 mutant showed total or almost total absence of phosphorylation in multiple experiments, suggesting that this mutation abolishes a major phosphorylation site.

In conclusion these data are consistent with a triple-T locus (106-108) representing a major tumor-specific phosphorylation locus of Apoptin.

7. Single point mutants confirm the Triple-T locus is a major phosphorylation site of Apoptin.

In order to demonstrate conclusively that the Triple-T locus contains a major phosphorylation site of Apoptin, we generated single point mutants, replacing T-106, T-107 and T-108 with an alanine, which cannot be phosphorylated. In addition, because there is a common family of kinases, known as the proline-directed kinases, that requires a proline directly downstream of the phosphorylation site (Ishida et al., 2000), we also replaced P-109 with an alanine (see Figure 4 for a summary of these constructs). The mutants were called, respectively, T106A, T107A, T108A, and P109A. Each of these constructs was transfected into Saos-2 cells, along with empty vector and CMV-VP3 controls, and subjected to the *in vivo* phosphorylation assay.

Whereas T108A and CMV-VP3 were still phosphorylated, T107A and P109A were not. T106A was faintly phosphorylated. Western blot analysis confirmed that all constructs were expressed at roughly equal levels. These results suggest that both T106 and T107 are necessary for Apoptin phosphorylation. Furthermore, the result with the P109A mutant suggests that the phosphorylation site resides within a consensus site of defined conformation, possibly mediated by a proline-directed kinase.

Thus we concluded that T106 and T107 are either both phosphorylated, or only one is and the other site is a necessary part of the kinase consensus sequence. Whichever is the case, both residues seem to be essential for the complete phenomenon, as is P109.

8. Evidence for a distal locus influencing the phosphorylation of Apoptin.

Interestingly, the Ala(5)-91 mutant (see Figure 3) reproducibly showed phosphorylation that was significantly reduced. Given that replacing residues 106-110 with alanines almost completely abolished Apoptin phosphorylation, whereas replacing residues 91-96 with alanines only impaired the phosphorylation somewhat, the most likely explanation is that the domain encompassed by 91-96 represents a facilitator of phosphorylation at the main triple-T locus. In contrast, if 91-96 merely represented a minor phosphorylation site, the triple-T knockout mutant should be more phosphorylated, and it is not. The presence of a phosphorylation-inducing domain in this locus is consistent with the fact that the GFP-100-121 mutant was reproducibly hypophosphorylated with respect to the other C-terminal fragments.

Taken together, these data are consistent with a model wherein the triple-T locus (threonines 106-108) contains a major tumor-specific phosphorylation site of Apoptin, whereas there might be a facilitator of phosphorylation within positions 91-95.

9. Apoptin phosphorylation within the triple-T locus is necessary for apoptosis induction in tumor cells

In order to determine the functionality of tumor-specific phosphorylation of Apoptin, we tested whether the alanine point replacement mutants described above could still induce apoptosis in tumor cells. In addition, we also prepared and tested mutants containing glutamic acid replacements at the key residues T106, T107, and as a control, T108 (see Figure 4). Glutamic acid is widely used to confer gain-of-function for mutated phosphorylation sites because the negative charge of this amino acid emulates the negative charge of the phosphorylation modification (Maciejewski et al., 1995). Note that the negative charge of the glutamic acid may be able to spread its effects to adjacent sites.

We inspected Saos-2 cells three to five days after transfection using immunofluorescence analysis. Cells expressing Apoptin constructs were scored as apoptotic if they contained a completely condensed, apoptotic nucleus. Wild-type Apoptin was able to kill tumor cells to a level that will be referred to here as the baseline level. In contrast, mutants T106A, T107A, and P109A induced reduced death compared to the baseline. Strikingly, the T106E and T107E mutants demonstrated a gain-of-function phenotype, in that the apoptosis induced was restored back to baseline levels by the glutamic acid substitution. Finally, the T108A and T108E mutant exhibited only baseline death. Thus, there was a correlation between the phosphorylation status of the mutant and the ability of that mutant to kill.

These results strongly support the hypothesis that phosphorylation occurs within T106-T107 and that this phosphorylation is necessary for the apoptotic function of Apoptin.

10. Domains influencing nuclear import and activity of Apoptin

We noticed that the Ala(5)-91 mutant was severely impaired for nuclear

import, as were the Ala(5)-81 mutant and Ala(5)-86 mutant. Furthermore, a GFP-100-121 fusion protein containing the C-terminal 22 amino acids of Apoptin did not accumulate in the nucleus, whereas a GFP-80-121 fusion protein containing the C-terminal 42 amino acids of Apoptin clearly did. In addition, the GFP-80-121 fusion protein induced also apoptotic activity in human tumor cells, such as Saos-2 cells.

The below-depicted Table 1 summarizes the results of the nuclear localization and induction of apoptosis by GFP-VP3 fusion proteins or Apoptin Ala-mutants. Both the GFP-VP3 and Apoptin Ala mutants have been described in Figure 2 and Figure 3, respectively.

Table 1

	Apoptin	
DNA-construct	nuclear localization	apoptosis
GFP-VP3	Yes	Yes
GFP-1-69	No	No
GFP-70-121	Yes	Yes
GFP-80-121	Yes	Yes
GFP-100-121	No	No
Ala(5)-81	No	ND
Ala(5)-85	No	ND
Ala(5)-91	No	ND
wild-type VP3	Yes	ND

ND not determined

Taken together these data with the phosphorylation data, it appears that the amino acids 80 to 121 comprises at least 3 key elements crucial for tumor-specific activity: nuclear import domain(s), phosphorylation within the triple-T locus, and a domain facilitating said phosphorylation. The said key elements will, for example, be essential to identify an universal mediator of phosphorylation in human tumor cells. Interfering with this mediator could provide a new anti-cancer therapy.

Description of the figures

Figure 1. The complete amino acid sequence of the Apoptin protein that is encoded by pCMV-VP3 and by the GFP-Apoptin constructs. (Note that the Apoptin protein encoded by the pIRESneo alanine mutants is a natural, phenotypically similar variant containing a K at position 116). Boxed underneath the primary sequence are various domains of Apoptin: 111.3, the epitope for monoclonal antibody 111.3; NES, the putative nuclear export signal; VP3-C, corresponds to the peptide used to raise the polyclonal antibody VP3-C; NLS1 and NLS2, the two putative nuclear localization signals in the potential bipartite arrangement. All potential conventional phosphorylation sites (serines, threonines and the sole tyrosine) are underlined.

Figure 2. A schematic representation of the GFP-fused mutant series of Apoptin. The sizes depicted are not to scale. White boxes, the green fluorescence protein (GFP) tag; grey boxes, the Apoptin fragment; black bars show the approximate location of the amino acid numbers on the boundaries of the deletions; striped boxes, a tether region between the GFP and Apoptin fragments; bent lines indicate where the deletion occurs.

Figure 3. A schematic representation of the 5-alanine linker-scanning mutant series of Apoptin. Only the region between 70 to 121 is shown. The amino acid sequence is shown on the top, including the NLS domains (boxed) for reference. The entire Apoptin sequence is identical (heavy black lines) except where the alanine replacements are depicted.

Figure 4. A schematic representation of the single point mutant series of Apoptin. Only the region between 70 to 121 is shown. The amino acid sequence is shown on the top, including the NLS domains (boxed) for reference. The entire Apoptin sequence is identical (heavy black lines) except where the

alanine or glutamic acid replacements are depicted. The mutated region between 106 and 109, inclusive, is underlined on the reference sequence.

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Claims

1. An isolated or recombinant phosphorylated Apoptin or functional equivalent and/or functional fragment thereof.

2. Phosphorylated Apoptin according to claim 1 or functional equivalent and/or functional fragment thereof wherein said Apoptin is tumor-specifically phosphorylated.

3. Phosphorylated Apoptin according to claim 1 or 2 or functional equivalent and/or functional fragment thereof wherein said Apoptin is phosphorylated on a threonine residue, which residue in the Apoptin of Figure 1 is located between amino acid 100 to 121.

4. Phosphorylated Apoptin according to anyone of claims 1 or 3 or functional equivalent and/or functional fragment thereof wherein said Apoptin is phosphorylated on a threonine residue, which residue in the Apoptin of Figure 1 is located at amino acid 106 and/or 107.

5. A vector comprising a nucleic acid encoding Apoptin or functional equivalent and/or functional fragment thereof, which can be phosphorylated according to anyone of claims 1 to 4 and further comprises a nucleic acid molecule encoding a kinase.

6. A gene delivery vehicle comprising a vector according to claim 5.

7. A host cell comprising a vector according to claim 5 or a gene delivery vehicle according to claim 6.

8. An isolated or synthetic antibody or functional equivalent and/or functional fragment thereof specifically recognising phosphorylated Apoptin according to anyone of claims 1 to 4.

9. An immunoassay comprising an antibody according to claim 8.

10.A nucleic acid encoding an antibody according to claim 8.

11. A vector comprising a nucleic acid according to claim 10.

12. A host cell comprising a nucleic acid according to claim 10 or a vector

according to claim 11.

13. Use of Apoptin or functional fragment thereof which can be phosphorylated according to anyone of claims 1 to 4 for diagnostic purposes.

14. A method for detecting the presence of cancer cells or cells that are cancer prone in a sample of cells comprising providing a cell lysate of said cells to Apoptin or functional equivalent and/or functional fragment thereof which can be phosphorylated according to anyone of claims 1 to 4 and determining phosphorylation state of said Apoptin.

15. A method for identifying a putative cancer-inducing agent comprising submitting a sample of cells to said agent, and detecting the presence of cancer cells or cells that are cancer prone in a sample of cells with a method according to claim 14.

16. A method for testing the *in vitro* treatment effect of Apoptin on tumor cells comprising providing a cell lysate of said tumor cells to said Apoptin or functional fragment thereof which can be phosphorylated according to anyone of claims 1 to 4 and determining phosphorylation state of said Apoptin.

17. A method according to claim 14 or 16 wherein said Apoptin further comprises a fusion protein.

18. A kit for detecting the presence of cancer cells or cells that are cancer prone comprising an antibody according to claim 8.

19. A kit for testing the *in vitro* treatment effect of Apoptin on tumor cells comprising an antibody according to claim 8.

20. A method for identification of tumor specific kinase comprising providing Apoptin or functional fragment thereof which can be phosphorylated according to anyone of claims 1 to 4.

21. A pharmaceutical composition comprising phosphorylated Apoptin according to anyone of claims 1 to 4, a vector according to claim 5, a gene-delivery vehicle according to claim 6 or a host cell according to claim 7.

22. A pharmaceutical composition according to claim 21 for the induction of apoptosis.

23. A pharmaceutical composition according to claim 22 wherein said apoptosis is p53-independent.

24. A pharmaceutical composition according to anyone of claims 21 to 23 for the treatment of a disease where enhanced cell proliferation or decreased cell death is observed.

25. A pharmaceutical composition according to claim 24 wherein said disease comprises cancer or auto-immune disease.

26. A method for treating an individual carrying a disease where enhanced cell proliferation or decreased cell death is observed comprising treating said individual with a pharmaceutical composition according to anyone of claims 21 to 25.

Title: Modifications of Apoptin

Abstract

The invention describes phosphorylated Apoptin. Apoptin is tumor-specifically phosphorylated and thereby part of the Apoptin apoptotic pathway in tumor cells is elucidated. The invention opens the way for new therapeutic possibilities, for example novel therapeutic compounds that can work alone or, sequentially to, or jointly with other known compounds. The invention further describes the use of tumor-specifically phosphorylation of Apoptin for diagnostic purposes. Such a diagnostic purpose can, for example, be a method for detecting the presence of cancer cells or cells that are cancer prone or a method to identify a putative cancer inducing agent or a method for the in vitro treatment effect of Apoptin on tumor cells by testing the phosphorylation state of Apoptin. Even more, the invention provides possibilities to further elucidate the apoptotic pathway and to identify for example crucial mediators of phosphorylation in human tumor cells. Interfering with such a mediator could provide new anti-cancer therapies.

Figure 1: The domains and potential phosphorylation sites of Apoptin

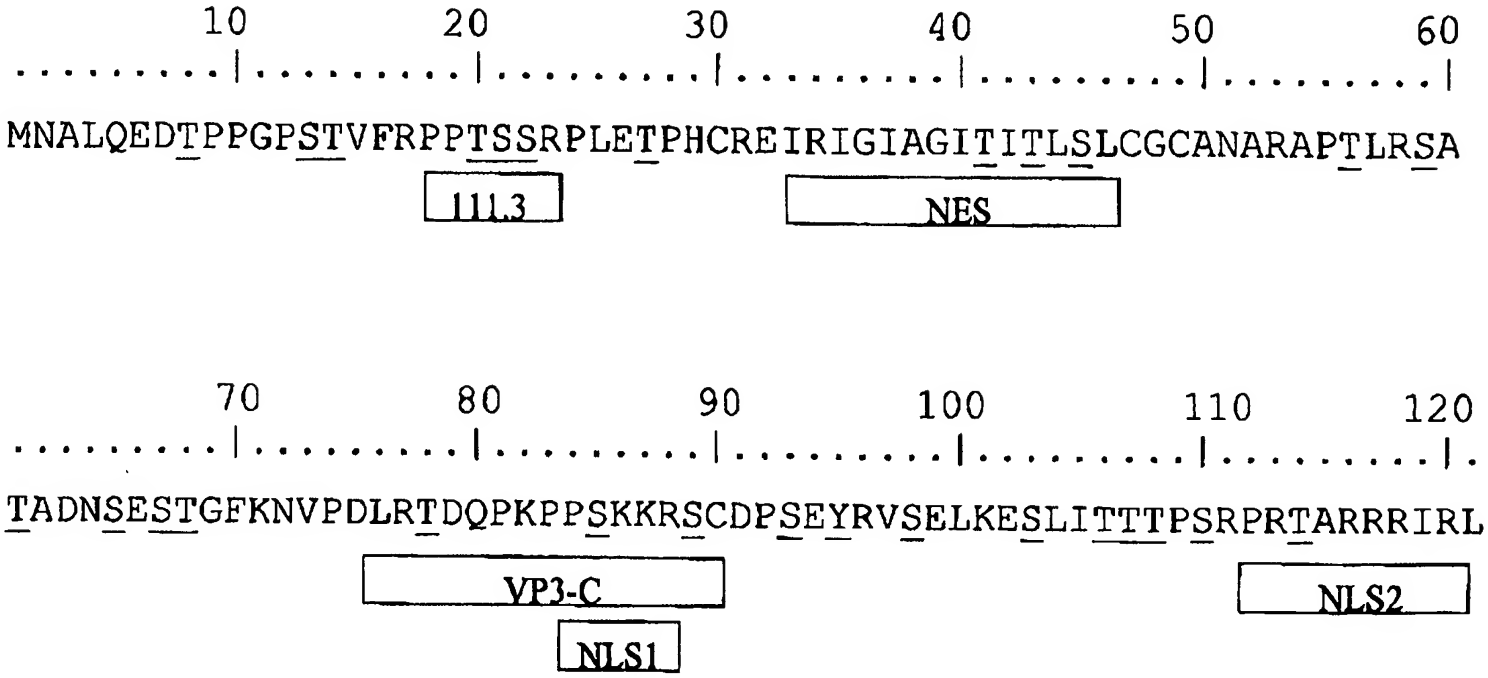

Figure 2: The GFP-fused mutant series of Apoptin constructs

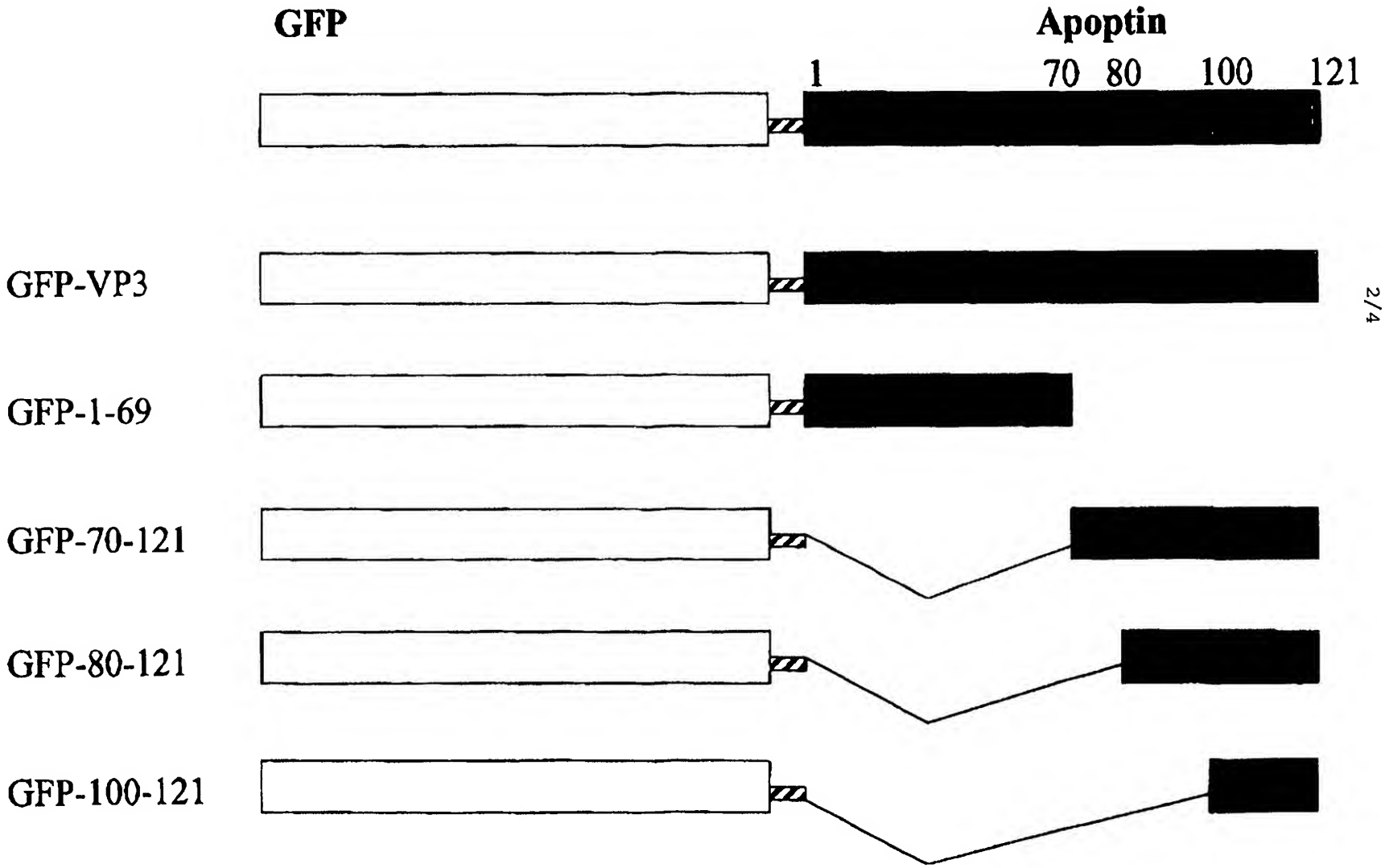

Figure 3: The 5-alanine linker-scanning mutant series of Apoptin constructs

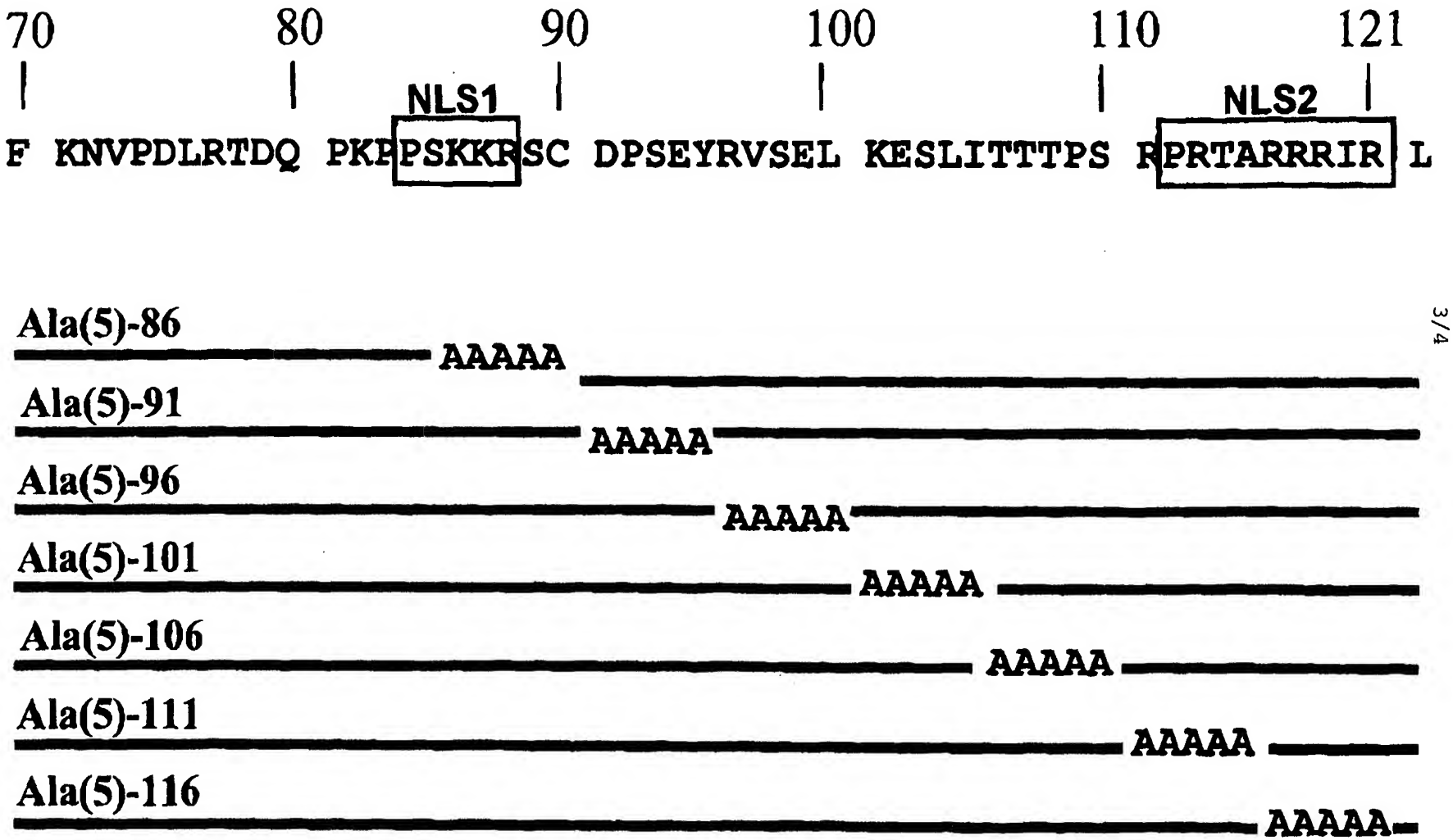

Figure 4: The single point mutant series of Apoptin constructs